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RESEARCH PAPERS - 9TH SPECIAL ISSUE ON GRAPEVINE TRUNK DISEASES

Pathogenicity of South African Hymenochaetales taxa isolated from esca-infected grapevines

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Summary. Little is known about the pathogenicity and etiology of Hymenochaetales taxa associated with esca in South Africa. Ten South African Hymenochaetales taxa associated with esca in grapevine were subjected to basic enzyme assays to determine which ligninolytic enzymes were secreted by each taxon. In addition, a field trial was undertaken to determine the pathogenicity of these taxa. Twenty-seven fungal isolates and two negative controls were inoculated into wounds made on mature grapevines of the cultivars Shiraz and Mourvédre. Inoculated vines were evaluated for white rot symptoms after 24 months. The results of the enzyme assays indicated a difference in enzyme secretion among taxa and also between isolates of the same taxa. All isolates secreted cellulase and laccase, but there was a difference in isolates' ability to secrete manganese peroxidase and lignin peroxidase. The results of the pathogenicity trial showed that all of the isolates used were capable of causing the characteristic white rot symptom in the wood. There were clear differences in susceptibility to white rot between the two cultivars tested, namely Shiraz and Mourvédre. The cultivars also differed in which taxa proved to be more virulent. On Shiraz a specific isolate of Taxon 6 (an *Inonotus* sp.), *Phellinus* sp. and *Inonotus setuloso-croceus* were significantly virulent. On Mourvédre, Taxon 3 (an *Inocutis* sp.) was significantly virulent.

Keywords: esca, grapevine trunk diseases, white rot, *Fomitiporia* sp.

Introduction

Field trials proving pathogenicity, i.e. the potential ability to cause disease or abnormalities in a host (Bos and Parlevliet, 1995), involving white rot are very rarely undertaken on grapevine or any other host. The result is that the etiology of the organisms that cause one of the defining features of mature esca is not understood very well. Differences in virulence, i.e. the severity of disease manifestation in infected individuals (Thomas and Elkington, 2004), between the species that cause white rot are not fully understood either. In literature, there have been four trials of varying sizes and complexity on mature vines (Chiarappa, 1997; Sparapano *et al*., 2000; Sparapano *et al*., 2001; Gatica *et al*., 2004) and two on young vines (Larignon and Dubos, 1997; Diaz *et al*., 2013). Additionally, one of the trials tested the rotting ability of *Phellinus* (*P*.) *punctatus* P. Karst. [now considered *Fomitiporia* (*F*.) *mediterranea* M. Fisch.] on wooden blocks (Larignon and Dubos, 1997).

Chiarappa (1997) successfully performed inoculations of *P. igniarius* (L.) Quél. on 7-year-old commercial vines and established *P. igniarius* as the main causal organism of the spongy decay symptom of the disease known as black measles in California. Larignon and Dubos (1997) inoculated *P. punctatus* on Cabernet Sauvignon cane segments, which were rooted for two months and grown in the glasshouse and the field for four months and a year, respectively, and wooden blocks taken from healthy Cabernet

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Sauvignon vines which were incubated for a year. The young vine inoculations of *F. mediterranea* in the same study showed brown vascular streaking, but the researchers were unable to re-isolate the basidiomycete from the inoculated plants. The wood blocks inoculated with *F. mediterranea* showed soft white rot after twelve months.

Sparapano *et al.* (2000) observed white rot symptoms two years after inoculating *F. mediterranea* on 13-year-old Sangiovese vines and, during inoculations on six- and nine-year-old Italia and Matilde vines, could detect the first signs of white rot after six months. Sparapano *et al.* (2001) included *F. mediterranea* in a cross-inoculation trial with *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & L. Mugnai and *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams on mature grapevines and found that *F. mediterranea* was able to cause limited, localised white rot within three years after inoculation. In Argentina, researchers performed a limited experiment with an undescribed *Phellinus* sp. associated with the Argentine grapevine trunk disease, "hoja de malvón" (Gatica *et al.*, 2004). This species was later identified as *Inocutis* (*Ic.*) *jamaicensis* (Murrill) A.M. Gottlieb, J.E. Wright & Moncalvo (Lupo *et al.*, 2006). Five 13-year-old plants were inoculated with the *Phellinus* sp. and showed internal and external symptoms of the disease within six years after inoculation. Diaz *et al.* (2013) attempted inoculation of a Chilean *Inocutis* sp. on axenic plantlets incubated for 28 days, rooted 2-year-old grapevines incubated for 15 months, grapevine shoots incubated for 60 days and detached grapevine shoots incubated for 14 days as part of a larger pathogenicity trial of several pathogens associated with esca in Chile. In the Chilean experiment, the *Inocutis* sp. caused brown vascular discolouration in all the inoculations; however, the incubation time of all the Chilean trials was considerable shorter than in the case of Sparapano *et al*. (2000) and Gatica *et al.* (2004).

White rot in wood is caused by the degradation of primarily lignin, but also cellulose and hemicellulose, within the wood cell-walls. Lignin and cellulose degradation are affected by extracellular enzymes released by wood rotting fungi, which break up the complex components of the cell wall (Manion, 1981). Lignin is a complex compound to degrade, and only white rot basidiomycetes have been found to do it efficiently (Songulashvili, 2006). Three enzymes have been found to be essential for lignin degradation, namely a copper containing phenoloxidase, laccase and two heme-containing peroxidases, lignin peroxidase (LiP) and manganese-dependent lignin peroxidase (MnP) (Overton *et al.*, 2006; Songulashvili, 2006). According to Morgenstern *et al.* (2010), it is unlikely that ligninolytic processes would be possible without production of either lignin peroxidase or manganese peroxidase. Past trials involving enzymatic assays and basidiomycetes involved with esca have shown that *P. igniarius* produces laccase and peroxidases and *F. punctata* (*F. mediterranea*) produces laccase and peroxidase (Chiarappa, 1959; Mugnai *et al.*, 1999). Having an indication of the types of enzymes secreted by the South African Hymenochaetales may provide some insight into the aetiology of these organisms, if not their pathogenicity.

South African vineyards are widely affected by trunk diseases, including esca (Van Niekerk *et al.*, 2011). White *et al.* (2011a) characterised ten novel basidiomycete taxa belonging to the order Hymenochaetales which were associated with white rot symptoms on vines affected by esca (White *et al.*, 2011b). The South African Hymenochaetales taxa associated with the esca disease complex represent several distinct genera. Four taxa could be identified based on morphological characteristics observed from fruit bodies, namely *Fomitiporella* sp., *Fomitiporia capensis*, *Phellinus* sp., and *Inonotus* (*In.*) *setuloso-croceus* (Cloete *et al.*, 2014; Cloete, 2015). The other taxa represent species from the genera *Fomitiporella* (Taxon 2), *Inocutis* (Taxon 3 and 4) and *Inonotus* (Taxon 5, 6 and 8) (White *et al*., 2011). The latter genus identifications were made due to a lack of suitable reference sequences on GenBank and no fruit bodies found. Given the diversity in species and genera associated with esca in South Africa, there is an expectation of variation in pathogenicity between different taxa. The main objectives of this study were i) to determine the ability of these taxa to induce white rot in mature vines by inoculating mature, externally asymptomatic vines in the field with all ten taxa and ii) to conduct basic *in vitro* enzymatic studies to determine which ligninolytic and cellulose-degrading enzymes were secreted by these taxa.

Materials and methods

Fungal isolates

Twenty-seven isolates representing ten South African Hymenochaetales taxa were selected from the collection used in White *et al.* (2011a) for pathogenicity testing (Table 1). The cultures are maintained in the culture collection of the Department of Plant Pathology at Stellenbosch University (STE-U). Two or three isolates (two in cases where only two isolates of a certain taxon were available) of every taxon originating from different grapevine cultivars and locations were selected for inoculation (Table 1). An isolate of *Acremonium strictum* W. Gams was selected as negative control. An uninoculated control consisting of uncolonised toothpicks and uncolonised Potato Dextrose Agar (PDA, Biolab, Merck, Gauteng, SA) was also used.Three weeks prior to inoculation, isolates were plated out on unamended PDA, and

Table 1. Hymenochaetales isolates from White *et al.* (2011a) used in a pathogenicity study conducted on 10-year-old vines between September 2010 and November 2012 in Stellenbosch.

Taxon	Isolate (STE-U number)	Origin	Cultivar isolated from
Fomitiporella sp.	7038	Stellenbosch	Sauvignon blanc
	7141	Riebeeck Kasteel	Chenin blanc
	7148	De Rust	Chenin blanc
Taxon 2 (*Fomitiporella* sp.)	7147	Oudtshoorn	Pinotage
	7154	Calitzdorp	Hanepoot
	7155	Calitzdorp	Hanepoot
Taxon 3 (*Inocutis* sp.)	7109	Constantia	Sauvignon blanc
	7136	Grabouw	Sauvignon blanc
	7174	Ashton	Sauvignon blanc
Taxon 4 (*Fomitiporella* sp.)	7042	Stellenbosch	Chenin blanc
	7043	Stellenbosch	Chenin blanc
Taxon 5 *(Inonotus* sp.)	7126	Darling	Chenin blanc
	7143	Tulbagh	Chenin blanc
	7153	Ladismith	Chenin blanc
Taxon 6 (*Inonotus* sp.)	7133	Malmesbury	Pinotage
	7134	Malmesbury	Pinotage
Inonotus setuloso-croceus	7090	Stellenbosch	Ruby Cabernet
	7106	Constantia	Sauvignon blanc
	7165	Franschhoek	Chenin blanc
Taxon 8 (*Inonotus* sp.)	7138	Botrivier	Chenin blanc
	7139	Botrivier	Chenin blanc
Fomitiporia capensis	7096	Franschhoek	Chenin blanc
	7135	Grabouw	Chardonnay
	7168	Hermanus	Chardonnay
Phellinus sp.	7055	Marken	Prime seedless
	7098	Kanon Eiland	Sultana
	7105	Marchand	Sultana

grown on triple-sterilised wooden toothpicks cut into 1 cm segments.

For the *in vitro* enzyme assays, the same isolates that were used in the pathogenicity study in addition to some reference isolates representing species associated with esca from other countries, as well as strains representing *P. igniarius* and *In. hispidus* were used (Table 2). Isolates were plated out on PDA plates two weeks before enzyme assays were carried out.

Enzyme assays

Enzyme assays for lignin peroxidase, manganese peroxidase, cellulase and laccase were performed on the 34 isolates indicated in Table 2. Plugs of 4mm diameter were cut from the margins of 2-week-old colonies representing all isolates and plated in triplicate on the respective media. Assays were repeated. For the manganese peroxidase, lignin peroxidase and laccase activity assays, negative reactions were scored as 0. Uncertain reactions, where one to five plates had a positive reaction, were scored as 0/1. Positive reactions, where all plates had a positive reaction, were scored as 1.

Manganese peroxidase

Mycelial plugs were plated onto a medium containing manganese sulphate at either 80 mg L^{-1} or 100 mg L^{-1} (Overton *et al*., 2006). Plates were incubated at 25°C for 20 days with 12 hour light-dark cycles. The presence of manganese peroxidase was indicated by a rust-coloured discolouration in the medium.

Lignin peroxidase

Mycelial plugs were plated onto a medium made up of 5% maltose, 1.4% agar and 0.03% anisidine (Sigma-Aldrich, Gauteng, SA) according to Conesa *et al*. (2000) and incubated for 14 days at 30°C. The production of peroxidase was indicated by a purple halo forming around colonies after the plates were flooded with a solution of 50 mM Na-tartrate buffer at pH 3, 50 μM H_2O_2 and 2 mM 3,4-Dimethoxybenzyl alcohol (Sigma-Aldrich, Gauteng, SA).

Cellulase

Mycelial plugs were plated onto a medium containing 0.5% carboxy-methyl-cellulose with 0.3% $NaNO_3$, 0.1% KH_2PO_4, 0.1% yeast extract and 0.05% $MgSO_4$ (St. Leger *et al*., 1997) and incubated at 25°C for 7 days. After incubation, staining and destaining was done with Congo Red (1 mg mL^{-1}) and 1 M NaCl, respectively. The isolates secreting cellulase formed a light halo caused by the cellulose degradation. This halo zone and the colony diameter were measured and expressed as a ratio of halo to colony diameter.

Laccase

Plugs were plated onto a 1.5% malt extract bacto agar medium containing 1% tannic acid (Merck) with an adjusted pH of 4.5. The presence of laccase was indicated by the medium turning brown (Rigling, 1995).

Pathogenicity trial

Site selection and plant material

Two vineyards in the Stellenbosch region of the Western Cape, South Africa, were selected for inoculation. Both vineyards were 10 years old, one Shiraz and the other Mourvédre. The vineyards were approximately 16 km apart. Prior to inoculation, vineyards were inspected for external symptoms of esca and dieback and were spot-tested for internal wood discolouration by cutting open randomly chosen, healthy-looking cordons. Suitable sites for inoculation were marked in advance. Inoculation sites were selected on asymptomatic vines and on well-developed cordons of more or less equal diameter, as far away as possible from existing pruning wounds and spurs.

Experimental design

The trial was laid out in a randomised block design. Experimental units consisted of one wound per vine and fungal treatments consisted of a single pathogen isolate. The negative controls consisted of uncolonised PDA and a non-pathogen control (*Acremonium strictum* STE-U 6296) (Damm *et al*., 2007). A total of 29 treatments were applied, representing multiple isolates of all ten basidiomycete taxa, a negative and an uninoculated control. Each treatment was replicated ten times.

Inoculations

During spring in September and October 2010, the two test vineyards were inoculated with selected fungal isolates. The inoculation method was similar to the method detailed in Sparapano *et al.* (2000), but adapted to be less harsh to vines and to minimise the risk of contamination. Each inoculation site was man-

Table 2. The results of assays testing for manganese peroxidase, lignin peroxidase and laccase activity on Hymenochaetales isolates from White *et al.* (2011a) as well as reference isolates.

Taxon	Isolate (MF or STE-U number)[a]	Manganese peroxidase[b]		Lignin peroxidase[c]	Laccase[d]
		80 ppm	100ppm		
Fomitiporia mediterranea 45/23	MF1	1	1	1	1
Fomitiporia australiensis 22485	MF2	1	1	1	1
Fomitiporia australiensis 22486	MF3	0/1	0/1	1	1
Phellinus alni TW 162	MF4	1	1	0	1
cf. *Fomitiporella vitis*, "Chile.I"	MF5	1	1	1	1
Fomitiporia polymorpha 91-42/2	MF6	1	1	1	1
Inocutis jamaicensis "ARG 10"	MF7	0/1	0/1	1	1
Phellinus igniarius 83-1022	MF8	0/1	0/1	0	1
Inonotus hispidus	MF9	1	0/1	0	1
Fomitiporella sp.	7038	0/1	1	1	1
	7141	0/1	1	1	1
	7148	1	1	1	1
Taxon 2 (*Fomitiporella* sp.)	7147	1	1	1	1
	7154	1	1	1	1
	7155	1	1	1	1
Taxon 3 (*Inocutis* sp.)	7109	0	0/1	0	1
	7136	0/1	0/1	0	1
	7174	1	0/1	0	1
Taxon 4 (*Fomitiporella* sp.)	7042	0	0	0	1
	7043	0	0	0	1
Taxon 5 (*Inonotus* sp.)	7126	0	0	0/1	1
	7143	0/1	0/1	1	1
	7153	0/1	1	1	1
Taxon 6 (*Inonotus* sp.)	7133	0	0	1	1
	7134	1	1	1	1
Inonotus setuloso-croceus	7090	1	1	1	1
	7106	1	1	1	1
	7165	0/1	0/1	1	1
Taxon 8 (*Inonotus* sp.)	7139	1	1	0/1	1
Fomitiporia capensis	7096	0/1	1	1	1
	7135	1	1	1	1
	7168	1	1	1	1
Phellinus sp.	7055	1	1	1	1
	7098	0/1	0/1	1	1
	7105	0/1	0/1	1	1

[a] MF reference isolates from the personal collection of Michael Fischer.
[b] Manganese peroxidase activity defined by 1=all plates discoloured, 0/1=one to five discoloured, 0=no plates discoloured.
[c] Lignin peroxidase activity defined by 1=all plates formed halo, 0/1= one to five plates formed halo, 0=no plates formed halo.
[d] Laccase activity defined by 1=all plates turned brown, 0/1=one to five plates turned brown, 0=no plates turned brown.

ually cleared of excess bark and sprayed with 70% ethanol solution. A 4 mm drill bit was used to drill 10 mm wounds into the wood. The drill bit was sterilised with 70% ethanol between inoculations. A colonised toothpick and a 1 cm^2 piece of colonised growth medium were inserted into each inoculation wound. Wounds were sealed with petroleum jelly (Vaseline, Unilever, SA) and covered with several layers of Parafilm (Bemis Flexible Packaging, Neenah, Wisconsin, USA). The inoculated vines were inspected at regular intervals for foliar symptoms.

Retrieval and sample processing

In October and November 2012, respectively, inoculations on the Mourvédre and Shiraz blocks were retrieved. A 30 cm piece of cordon around each wound site was removed and immediately taken to the laboratory. Cordon pieces were stripped of excess bark and split lengthwise through the inoculation site with a bandsaw (Toolmate, DT group, Denmark). All internal discolouration lengths and wound sites were measured and photographed. Any wood rot found to occur was measured lengthwise. Samples were triple sterilised in 70% ethanol (30 s), undiluted bleach (NaOCL) (2 min) and 70% ethanol (30 s) and left to dry in the laminar flow cabinet. Isolations from internal symptoms were made at five positions, the first at the proximal end of the symptom, the second in the middle of the symptom, the third at the inoculation wound site, the fourth in the middle of the symptom on the distal side of the wound and the fifth on the distal end of the symptom. Five wood pieces were extracted from every isolation point. Isolated wood pieces were placed on PDA plates amended with chloramphenicol (250 mg per plate) and incubated on the lab bench at 23–25 °C. Emerging basidiomycete colonies were sub-cultured and kept for identification.

Molecular identification of isolated colonies

Basidiomycete cultures resulting from the trial plates were identified with species-specific primers (Bester *et al*., 2014). Isolates of which the identity could not be confirmed by this protocol were cloned and sequenced according to the protocol detailed in Cloete *et al*. (2014).

Statistical processing

Pathogenicity, defined in this study as the ability of the fungal agent to cause white rot as a primary rot-inducing agent, was calculated by the measurement of white rot occurring on inoculated vines compared to the negative and uninoculated controls to ascertain which isolates and taxa could be classified as pathogenic compared to controls. Lesion lengths, defined as any dark discolouration surrounding the point of inoculation, were also measured in order to ascertain whether there was a significant difference in internal discolouration formed between controls and treated wounds. The data were subjected to analysis of variance (ANOVA) and the means were compared by Fischer's least significant difference (LSD) with P=0.05. Analysis was performed using SAS 9.2 (SAS Institute Inc, Cary, North Carolina, USA). The incidence rates were calculated according to the absence or presence of white rot at the inoculation site and calculated as a percentage of the total number of vines inoculated with that particular isolate. The re-isolation percentages were calculated as a percentage of the inoculated basidiomycete recovered from isolation.

Results

Enzyme assays (see Table 2)

Manganese peroxidase

All isolates representing Taxon 2 and the single isolate of Taxon 8 were able to produce manganese peroxidase (MnP). *Fomitiporella* sp., Taxon 3, Taxon 6 and *In. setuloso-croceus* had variation between isolates with some displaying positive, some negative and some uncertain results. Taxon 4 and a single isolate of Taxon 5 (STE-U7126) did not produce MnP. *Fomitiporella vitis, P. alni, F. polymorpha, and F. mediterranea* had positive results for MnP activity. *Fomitiporia australiensis* had mixed results between the two isolates tested. *Inocutis jamaicensis*, *P. igniarius* and *In. hispidus* had uncertain results.

Lignin peroxidase

All reference isolates could produce lignin peroxidase (LiP), with the exception of *P. alni*, *P. igniarius* and *In. hispidus*. All isolates representing *Fomitiporella* sp., Taxon 2, Taxon 6, *In. setuloso-croceus*, *F. capensis* and *Phellinus sp.* could produce LiP, as could two of the Taxon 5 isolates. One of the three Taxon 5 (STE-U 7126) isolates and the Taxon 8 isolate had uncertain results. None of the Taxon 3 or Taxon 4 isolates were able to produce LiP.

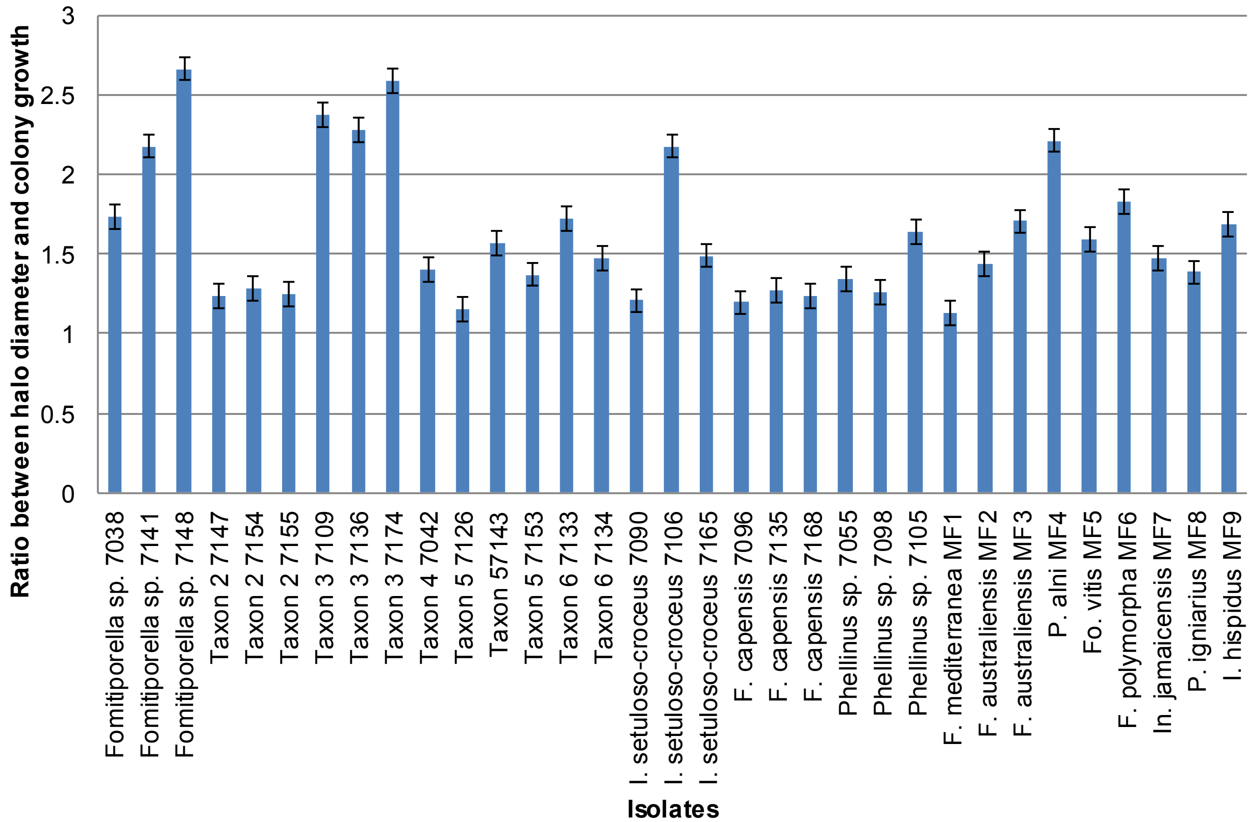

Figure 1. Ratio of halo diameter to colony growth obtained during the cellulase assays on the reference isolates and Hymenochaetales isolates from White *et al.* (2011a). Bars represent the standard error of the mean between repeats within experiment.

Laccase

All isolates were able to produce laccase.

Cellulase

All isolates tested were able to produce cellulase (Figure 1). Two isolates of *Fomitiporella* sp. (STE-U7141, STE-U7148), all the isolates of Taxon 3, one isolate of *In. setuloso-croceus* (STE-U7106) and the *P. alni* reference isolate produced a halo, with a colony size ratio of more than two. Only four isolates, one representing Taxon 5 (STE-U7126), one *In. setuloso-croceus* (STE-U7090), one *F. capensis* (STE-U7096) and the reference isolate for *F. mediterranea* produced ratios smaller than 1.25.

Pathogenicity trials

None of the inoculated vines produced external symptoms associated with esca or other trunk diseases during the 2 year incubation period.

Inside the wood, the symptom types were evaluated separately as brown discoloured lesions and white rot. All inoculated samples displayed an ellipse-shaped interior discolouration with the broadest area around the wound site (Figure 2). Discoloured tissue was dark brown to black. In some samples, light to dark yellow rotted tissue could be observed (Figure 2). Rotten tissue was soft, spongy and moist to the tuch.

Lesions: The lengths of the dark discoloured tissue lesions on all inoculated plants did not differ significantly from the control lesions in Shiraz, and there were no significant differences between taxa ($P=0.6340$) or between isolates within taxa ($P=0.3978$). In Mourvédre, there was a significant difference between taxa ($P<0.0001$), though not between isolates within taxa ($P=0.7951$) and only Taxon 3 was able to form lesions which differed significantly from

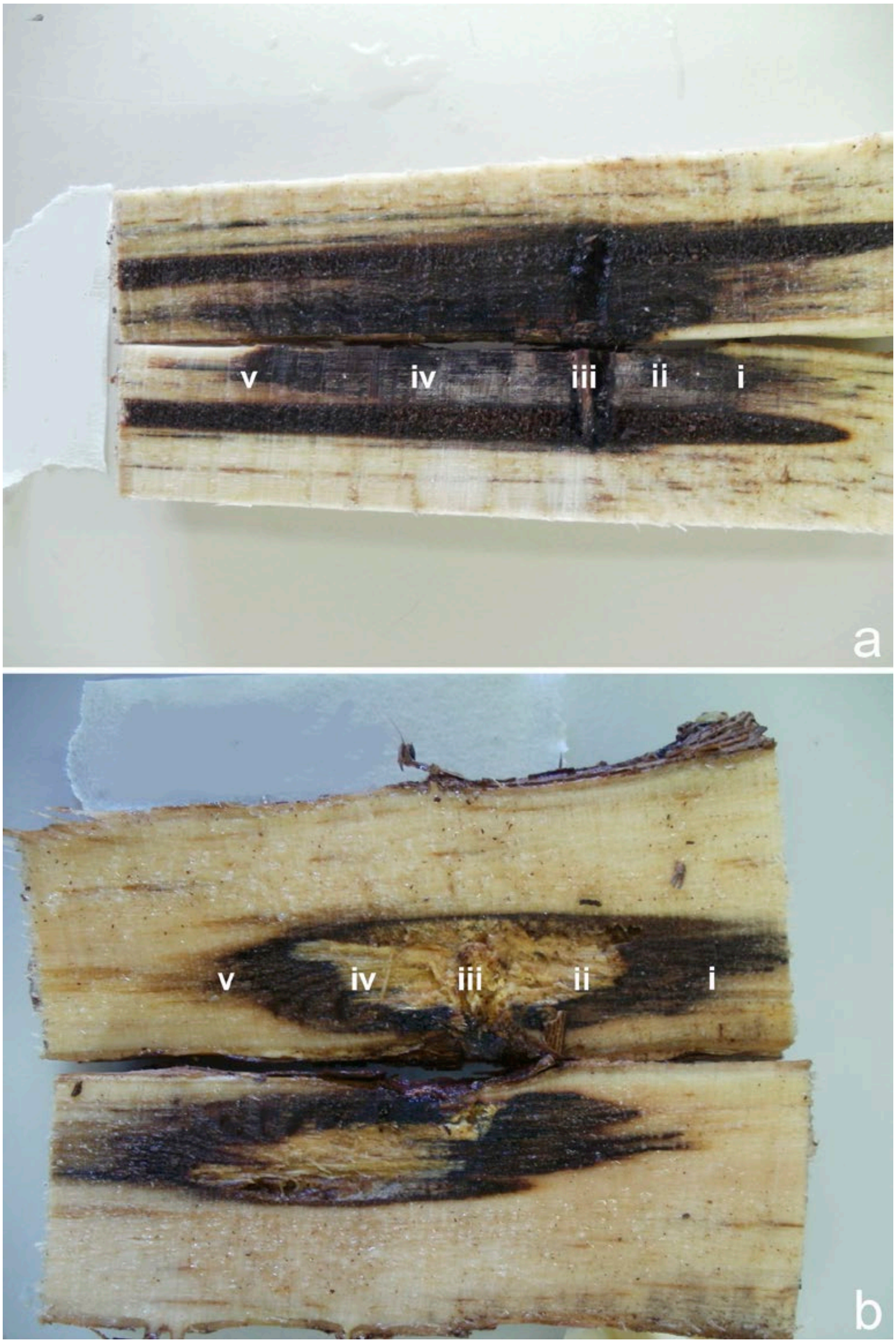

Figure 2. Isolation sites on Hymenochaetales inoculated grapevine samples. a. A discoloured lesion without rot on Shiraz. i. Isolation site on the proximal end of the lesion. ii. Isolation site in the middle of the lesion. iii. Isolation at wound site. iv. Isolation site in the middle of the lesion. v. Isolation site on the distal end of the lesion. b. A discoloured lesion with white rot on Mourvédre, i-v the same as on Shiraz.

Table 3. Mean lengths of brown discoloured lesions (mm) produced in the cordons of Mourvédre vines inoculated with representative isolates of Hymenochaetales taxa.

Mean	No.*	Taxon
65.67[a]	30	Taxon 3
37.51[b]	10	Negative control
36.58[bc]	20	Taxon 4
33.55[bc]	30	*I. setuloso-croceus*
32.65[bc]	30	*Fomitiporella* sp.
31.55[bc]	30	Taxon 5
29.35[bcd]	30	*F. capensis*
28.68[bcd]	18	Taxon 8
28.15[bcd]	30	Taxon 2
26.32[bcd]	30	*Phellinus* sp.
24.47[cd]	20	Taxon 6
18.30[d]	10	Uninoculated control
LSD (*P*=0.05) 12.202		

[a-d] Values within a column followed by the same letter are not significantly different.
* Number of vines analysed.

the control (Table 3). Multiple isolations were made from five points along the length of discoloured internal tissue. During reisolation, inoculated cultures could be recovered from all points of isolation.

White rot: The results of the pathogenicity trial showed that all of the isolates were capable of causing the characteristic white rot symptom in the wood to some extent, though not in every inoculated plant. None of the control plants had white rot. The extent of white rot observed on Shiraz (0.2–5.8 mm) was significantly less than that observed in Mourvédre (1.4–40.9 mm). In Shiraz, there was a significant difference between isolates within a taxon (P=0.03), and most isolates did not differ significantly from the controls (Table 4). Single isolates of Taxon 6 (STE-U 7133), *In. setuloso-croceus* (STE-U 7090) and *Phellinus* sp. (STE-U 7055) proved significantly virulent. In Mourvédre, there was no significant difference between conspecific isolates (P=0.1838). There was variation between taxa with regard to rot lengths (P<0.0001) with Taxon 3 and Taxon 2 proving significantly virulent (Table 5). During re-isolation from Shiraz, 26.89 % of isolates were recovered from inoculated vines. In Mourvédre, 65.17 % of isolates were recovered from inoculated vines.

Table 4. Mean (Least Squares) white rot lengths (mm) between different Hymenochaetales taxa inoculated into Shiraz vines.

LSMean	Taxon	Isolate
10.08[a]	Taxon 6	STE-U7133
7.96[ab]	*Inonotus setuloso-croceus*	STE-U7090
6.52[abc]	*Phellinus* sp.	STE-U7055
3.77[bcd]	Taxon 3	STE-U7109
3.47[bcd]	*Fomitiporella* sp.	STE-U7148
3.01[dc]	Taxon 8	STE-U7139
2.71[dc]	Taxon 5	STE-U7143
2.48[dc]	Taxon 5	STE-U7126
2.45[dc]	*Phellinus* sp.	STE-U7105
2.16[dc]	Taxon 5	STE-U7153
2.13[dc]	*Phellinus* sp.	STE-U7098
2.02[dc]	Taxon 6	STE-U7134
1.55[d]	Taxon 3	STE-U327
1.45[d]	*Inonotus setuloso-croceus*	STE-U7090
1.44[d]	*Fomitiporella* sp.	STE-U7141
1.43[d]	Taxon 8	STE-U7138
0.91[d]	*Fomitiporia capensis*	STE-U7135
0.78[d]	Taxon 3	STE-U7136
0.75[d]	*Inonotus setuloso-croceus*	STE-U7165
0.70[d]	*Fomitiporella* sp.	STE-U7038
0.69[d]	Taxon 2	STE-U7155
0.60[d]	Taxon 4	STE-U7042
0[d]	Uninoculated control	Uninoculated control
0[d]	*Fomitiporia capensis*	STE-U7168
0[d]	*Fomitiporia capensis*	STE-U7096
0[d]	Taxon 2	STE-U7154
0[d]	Taxon 2	STE-U7147
0[d]	Taxon 4	STE-U7043
0[d]	Negative control	Negative control

[a-d] Values within a column followed by the same letter are not significantly different.

Table 5. Mean white rot lengths (mm) produced in the cordons of Mourvédre vines inoculated with representative isolates of Hymenochaetales taxa.

Mean	No.*	Taxon
40.87[a]	30	Taxon 3
12.01[b]	30	Taxon 2
11.07[bc]	30	*Fomitiporella* sp.
10.14[bc]	29	Taxon 5
7.28[bc]	29	*Phellinus* sp.
7.17[bc]	29	*I. setuloso-croceus*
6.22[bc]	20	Taxon 4
5.40[bc]	26	*F. capensis*
3.57[bc]	19	Taxon 6
1.43[bc]	18	Taxon 8
0[c]	7	Uninoculated control
0[c]	8	Negative control
LSD (P=0.05) 11.563		

[a-d] Values within a column followed by the same letter are not significantly different.
* Number of vines analysed.

Discussion

Based on the results of the basic enzyme assays, variation in virulence between isolates and taxa may manifest in the array of ligninolytic enzymes secreted by the various taxa. The reference species, chosen for their documented ability to cause white rot on various hosts, also displayed variation in the types of enzymes secreted. Although all isolates were able to produce laccase and cellulase, there was variation between taxa in terms of their ability to produce manganese peroxide and lignin peroxidase, two critical enzymes in lignin degradation (Overton *et al.*, 2006; Morgenstern *et al.*, 2010). All the South African taxa could produce either lignin peroxidase or manganese peroxidase to a certain extent, except for Taxon 4, an *Inocutis* sp., which could produce neither. Only two South African taxa could not produce any lignin peroxidase, namely the two putative *Inocutis* species (Taxon 3 and Taxon 4). *Ic. jamaicensis*, the reference isolate, produced both manganese peroxidase and lignin peroxidase. Among the reference isolates, the two *Phellinus* species, *P. alni* and *P. igniarius* as well as *In. hispidus* could not produce lignin peroxidase, but could produce manganese peroxidase to varying degrees. Unlike *P. alni* and *P. igniarius*, all isolates of the South African based *Phellinus* sp., produced lignin peroxidase. Based on Morgenstern *et al.* (2010)'s assertion that lignin degradation is not efficiently achieved by laccases alone, and that peroxidases are necessary for the process to occur, there is an expectation that Taxon 4, an *Inocutis* species, would not be able to cause extensive white rot within a short period of time. More detailed investigation into the enzymes secreted by novel Hymenochaetales species is needed.

The occurrence of any white rot in inoculated samples showed that all of the South African Hymenochaetales taxa are pathogenic and have potential to cause white rot symptoms on mature commercial vines within two years. Due to the relatively short incubation time the extent of the rot development was not always significantly different from the controls due to the small amounts of rot formed. In comparison, Gatica *et al.* (2004) and Chiarappa (1997) left inoculated plants in the field for six and eight years, respectively. Sparapano *et al.* (2000) concluded that *F. mediterranea* could be considered a primary pathogen after observing white rot symptoms within two years. Several valuable observations may be gleaned from the data in this current study, as these trials are rarely undertaken on such a scale.

The more virulent taxa differed between Shiraz and Mourvédre. On Shiraz, specific isolates of Taxon 6, an *Inonotus* sp., *Phellinus* sp. and *In. setuloso-croceus* could be considered more virulent. Taxon 3, an *Inocutis* sp., was the only significantly virulent taxa on Mourvédre. There was no significant difference between conspecific isolates in the Mourvédre block.

These differences in virulent taxa between cultivars could be ascribed to, among other factors, differences in enzyme profiles between taxa as discussed in the previous section, fungal suitability to colonisation of the particular substrate and various physiological differences between the two cultivars. Cultivar differences in sensitivity to grapevine trunk diseases have been subject to several studies (Peros and Berger, 1994; Sosnowski *et al*., 2007). Apart from Sparapano *et al*. (2000) that showed that cultivar Matilde was less susceptible to white rot (*F. mediterranea*) than the cultivar Italia, very little is known about grapevine cultivar susceptibility towards white rot. Mourvédre is a cultivar thought to be particularly susceptible to esca while not being particularly sensitive to other trunk diseases (McGourty, 2003). Presumably, cultivar differences in sensitivity may be due to plant defenses, such as the formation of polyphenols which inhibit peroxidases and phenoloxidases (Del Rio *et al.*, 2004). Cultivar differences may also be due to physical factors such as differences in wood density between cultivars, a phenomenon that is easily observable in the field, though not documented in detail. Recent work suggests that cultivar differences in xylem morphology play a part in the ease of fungal colonisation (Pouzoulet *et al.*, 2014). Physiological factors also play an important role in plant resistance against trunk disease. During a study on pruning wound protection, Rolshausen *et al.* (2010) demonstrated that a cultivar with a documented susceptibility to trunk disease, Cabernet Sauvignon, had lower lignin content than a tolerant cultivar, Merlot, making it easier for pathogens to physically penetrate the grapevine wood. It stands to reason that factors such as lignin content will influence, not only the ability of pathogens to penetrate wood, but also the rapidity of development of symptoms such as white rot.

Grapevine wound response is a complex process consisting of many factors. Among these factors is the creation of physical barriers to prevent colonisation and spread by pathogens. Cell walls are fortified with additional lignin and pectin, tyloses and gums are formed and enzyme-inhibiting phenolic compounds accumulate around the infected zone to slow down the spread of infection (Del Rio *et al.*, 2001; Edwards *et al.*, 2007; Mutawila *et al.*, 2011). In Sparapano *et al.* (2000), a discolouration similar to the one found in the current trial was formed on either side of inoculations; however, they recorded a significant difference between inoculated and uninoculated vines. During the current trial, the length of internal discolouration surrounding all inoculations was measured. There was little to no variation between taxa in terms of lesions formed. On Shiraz, the most striking result was the fact that the negative and uninoculated controls didn't form lesions statistically different from most of the fungal taxa inoculated. In field inoculated vines of Sangiovese, Sparapano *et al*. (1999) also found similar brown discolouration on the control and *F. mediterranea* inoculated vines. It would seem that the brown discolouration formed was due to wounding. On Mourvédre, Taxon 3 was the only fungal inoculation to form lesions that were statistically longer than the negative and uninoculated controls; on their part, the controls didn't differ significantly from the other fungal taxa treatments. The fact that one taxon was significantly different from the controls may indicate that Mourvédre has a less robust response to wounding than Shiraz and may explain the difference in the extent of white rot found in both cultivars. If fungal isolates recovered after a two year period can be interpreted as a possible reflection of the host's ability to prevent colonisation, the percentage of isolates recovered from Shiraz (26,89%) compared to those recovered from Mourvédre (65,17%) could be seen as an indication of the efficacy of the cultivar Shiraz's short term defences.

As in the case of Bos and Parlevliet (1995), pathogenicity of white rot-causing organisms should be defined as the ability of a species to form white rot. This trial demonstrated that all South African Hymenochaetales taxa have the potential to be primary inducers of white rot on grapevine to varying degrees, given enough time and the right circumstances. Taxa, and isolates within the same taxa, vary in their ability to produce enzymes, as well as their ability to produce rot in the host. There were dramatic differences between the two cultivars tested in terms of their susceptibility to white rot, which will play a role in their overall susceptibility to esca in the long run.

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